Pecten as a new substrate for IcPD dating: The quaternary raised beaches in the Gulf of Corinth, Greece

Pecten as a new substrate for IcPD dating: the Quaternary raised beaches in the Gulf of Corinth, Greece.

Federica Pierini[a], Beatrice Demarchi[b]*, Jenni Turner[c], Kirsty Penkman[d]

[a] Department for Evolutionary Ecology, Max Planck Institute for Evolutionary Biology, 24306 Plön, Germany. pierini@evolbio.mpg.de.

[b] BioArCh, department of Archaeology, University of York, YO10 5DD, UK. beatrice@palaeo.eu. Tel: +44 (0) 1904 328559.

[c] School of Environmental Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ. UK. jenni.turner@uea.ac.uk.

[d] BioArCh, Department of Chemistry, University of York, YO10 5DD UK. kirsty.penkman@york.ac.uk.

*Corresponding author

Abstract

Intra-crystalline protein diagenesis (IcPD), a recent development of amino acid racemization dating (AAR), is now established as a reliable geochronological tool for the Quaternary. However, extending the method to new biominerals requires extensive testing in order to provide evidence for the closed-system behaviour of the intra-crystalline proteins and to assess the temporal span that can be covered.

Here we present results from high-temperature experiments on the IcPD of the bivalve *Pecten*, demonstrating that a fraction of proteins can be isolated from a bleach-resistant mineral matrix, which effectively operates as a closed system under conditions of accelerated diagenesis in the laboratory. Analyses of *Pecten* from the well-dated terrace system of the Gulf of Corinth (Greece) provided a pilot test for the integrity of the intra-crystalline fraction in subfossil shells. The small sample sizes in this preliminary study preclude a full assessment of the aminostratigraphic power of *Pecten* IcPD, but a concordance is observed between the extent of IcPD and sites dating from between MIS 5 and MIS 11.

We conclude that *Pecten* is a potentially good substrate for IcPD dating in the Mediterranean, and that the temporal limit of the technique in this area lies beyond MIS 11.

Highlights

- A 48-h bleaching step isolates the intra-crystalline fraction of proteins from the modern marine bivalve *Pecten*.
- The intra-crystalline proteins behave as an effectively closed system during artificial diagenesis.
- The patterns of diagenesis are predictable, but may differ between high temperature experiments and normal burial temperatures.
- The extent of IcPD was determined for *Pecten* within a restricted geographical area (the Gulf of Corinth, Greece).
- There is a concordance between the extent of IcPD and correlated age estimates back to MIS 11 in this region, although the resolution decreases beyond MIS 7.

Keywords

Intra-crystalline proteins; *Pecten* (scallop) shell; amino acid racemization (AAR); dating; raised beaches; Mediterranean.

1. Introduction

1.1 A new substrate for IcPD dating

Several studies have used amino acid racemization (AAR) geochronology to obtain age information for Quaternary raised beaches in the Mediterranean basin (Hearty, 1986, 1987; Hearty et al., 1986; Belluomini and Delitala, 1988; Hearty and Dal Pra, 1992; Torres et al., 2000, 2010). We have recently reported on the suitability of the bivalve *Glycymeris* for intra-crystalline protein diagenesis (IcPD) dating, concluding that while bleaching isolates an intra-crystalline protein fraction, this fraction may not operate as a closed system in fossil samples of this taxon, identified by comparing the diagenetic patterns of free and total hydrolysable (FAA vs THAA) amino acids (Demarchi et al., 2015). As part of the same study on IcPD dating of Mediterranean shorelines ("mAARiTIME"), we tested a new substrate, the bivalve *Pecten*, which had been successfully used by Murray-Wallace and colleagues (1996; 2005) to build AAR chronological frameworks using whole shell (i.e. unbleached) back to MIS 8 for low shelf deposits in Australia.

The attraction of this substrate lies in the fact that scallop shells are made of calcite and therefore the issues associated with aragonite diagenesis and recrystallization to calcite can be circumvented (Penkman et al., 2013). One further advantage of using *Pecten* as a substrate for IcPD dating is that some protein sequence data for this taxon are available and, in future, this could allow in-depth studies of the patterns of protein degradation using a combined approach of mass spectrometry and bulk amino acid analyses (e.g. Demarchi et al., 2013a).

The overall aims of our study were to assess if *Pecten* could provide a potentially reliable substrate for IcPD dating and to test the temporal span of the technique in the Mediterranean. Here we present results obtained on both modern (experimental) shells and fossils from raised terraces in the Gulf of Corinth, Greece.

As for all studies aimed at establishing the closed-system behaviour of a molluscan species under conditions of natural and accelerated diagenesis, we performed:

a. Bleaching experiments, to optimise the isolation of an operationally-defined intra-crystalline fraction of proteins.
b. High-temperature experiments, to test the integrity of the intra-crystalline fraction under continuous leaching conditions and the predictability of the patterns of diagenesis (closed-system behaviour).
c. Pilot studies on a limited number of fossil shells which were available from geological exposures with good independent age information, to test the suitability of the substrate for dating applications.

2. Materials and methods

2.1 Study site: Gulf of Corinth (Fig. 1)

The Gulf of Corinth (GoC) is an active rift in the eastern Mediterranean (Lat. 38°12'0" N, Long. 22°30'0" E), connected at its western end via a narrow straight to the Ionian Sea. The eastern and southern coastline of the GoC is on the footwall of active normal faults, which (with a small component of regional uplift (see Turner et al., 2010)) results in sediments deposited at the margins of these coastlines being raised to at least 200 m above sea level (asl). Uplift of marine sediments in the eastern side of the Gulf created an isthmus, through which excavations carried out between 1881 and 1893 cut the Corinth Canal, thus exposing extensive Quaternary stratigraphic sections, 5.8 km in height and up to 85 m in height. These include fossiliferous shallow marine facies of Holocene to MIS 11 age (Collier, 1990). Similar facies are particularly well preserved and exposed at intervals around the Perachora Peninsula, which lies north and north-west of the Corinth Canal (Fig. 1). Uplift of marine facies includes *in situ Cladocora caespitosa* coral stems, which have been dated by uranium-thorium (U-Th). The age of these corals allowed calculation of uplift rates (e.g. Vita-Finzi and King, 1985; Collier 1990; Dia et al., 1997; Morewood and Roberts, 1999; Leeder et al., 2003, 2005; Roberts et al., 2009; Turner et al., 2010) and provided age control for associated fossil material sampled from the same units.

2.2 Sample ages and locations

This study used fossil *Pecten* shells collected *in situ*, adjacent to dated coral colonies on the isthmus and Perachora Peninsula (e.g. Fig. 2B). Several coral stems from each location yield U-Th ages of the same marine oxygen isotope stage (MIS) highstand, giving robust age control for the associated *Pecten* shells dated to between MIS 5a/c and at least MIS 11 (Table 1). The terminology “MIS ages” is used here to indicate correlated age estimates, obtained by independent dating of the marine oxygen isotope stages. In order to define an aminozone, at least 3 individual shells should be analysed (Miller and Hare, 1980), and therefore this paper only presents a feasibility study on the potential utility of *Pecten*.

All *Pecten* shells (identified only to genus level) were collected between 2004 and 2008 by one of us (JT), prior to the IcPD study. *Pecten* and *C. caespitosa* were typically encased in marls with up to 10 m thickness of overlying semi-lithified marine and near-shore sediments (e.g. Fig. 2A). Therefore, although the initial purpose of the shell collection was not for an AAR study, the thickness of the sediments minimises the effect of surface temperature on the rates of diagenesis (Wehmiller, 1977; 1982). The shells were subsequently stored in sealed bags in boxes in rock store room at stable temperatures. Only *in situ* shells that were closely associated with dated coral stems were carefully selected for the IcPD study.

This contemporaneous fauna lived in the shallow marine zone at depths which typically ranged from 1 to 20 m below sea level, but over time were buried and preserved by marl (calcareous muds and silts) as sediments accumulated on the sea bed. As the coastline prograded and aggraded, shallow marine sediments were overlain by near-shore and then beach deposits (Fig. 2A). Seawards of the beach the sea-bed formed a gentle gradient dipping off-shore; over time marine sediments, including marine fauna, accumulated on the near-shore seabed during the highstand of each MIS warm stage. Where the coastal zone lay on the footwall of active faults, such as the on the Perachora peninsula, these coastal and near-shore deposits were subsequently uplifted. Footwall uplift continued during MIS cold-stages, when eustatic sea level fell; with sea level rise at the subsequent (younger) MIS warm stage, the marine deposits of the previous highstand were now raised above this new sea level. This

results in the modern coastal landscape being a flight of MIS warm-stage marine terraces (former sea beds) now at increasing elevation with increased age (Leeder et al., 2003). The terraces with exposures of underlying marine facies are particularly well-preserved on the Perachora Peninsula (Fig. 1C), with palaeo-beaches raised to ~75 m (MIS 7), ~30 m (MIS 5e) and ~12 m (MIS 5a/5c) above modern mean sea level (Leeder et al., 2003; Turner et al., 2010). Man-made cuttings through the terraces expose underlying highstand deposits. Some uncover a transition from shallow marine facies to near-shore and then beach sediments as a vertical transition, which records a prograding shoreline (e.g. Fig. 2A). Other exposures (such as at Agriliou Bay) are sea-to-landward cross-sections, revealing an inclined lateral transition of contemporaneous from shallow marine facies to beach facies.

Most shells considered here were collected from locations around the Perachora Peninsula, but in order to extend the study beyond MIS 7, we also analysed *Pecten* shells collected from the Corinth Canal (adjacent to corals dated to MIS 9 and 11 (Collier, 1990)). The various sample locations (Fig. 1C) and ages (Table 1) are summarised below:

MIS 11: A *Pecten* shell Gr 5 was collected from ~70 m elevation on the north side of the Corinth canal (Fig. 2A and B). Associated corals were dated to >350 ka by U-Th (this represented the upper limit of the technique at the time) and correlated with MIS 11 by Collier (1990).

MIS 9: Shell Gr 6 and associated corals were collected at ~30 m elevation on the north-west side of the canal; the corals were U-Th dated to 311.8 +33.4/-25.8 ka (MIS 9) by Collier (1990).

MIS 7: Shell Gr 7 is from the south-east end the Corinth Canal and was collected from ~15 m elevation (this location has been down-faulted since MIS 6); corals from this location were U-Th dated to 205.2 +13/-11.7 ka by Collier (1990).

MIS 5e: There are several *Pecten* shells from MIS 5e.

- The corals and associated *Pecten* shell Gr 8 were collected at the 25 m palaeoshoreline of Makrugoaz Ridge. The coral U-Th ages range from 108.5±0.7 to 134.0±3.0 ka and were attributed to MIS 5e in Leeder et al. (2003).
- The corals and associated *Pecten* shell Gr 13 were collected at ~ 23 m elevation, from the New Corinth Terrace. A coral sample was U-Th dated to 132.1 +8.2/-7.5 ka and correlated with MIS 5e in Leeder et al. (2005).
- The corals and associated *Pecten* shells Gr 14 and 15 were collected at ~ 10 m elevation at Agriliou Bay. Corals from this location are dated to MIS 5e by Dia et al. (1997) but to MIS 5 to 9 in Roberts et al. (2009). However, a MIS 5e age is considered reliable, as the fossiliferous unit is clearly associated with lateral transition to beach sediments at ~30 m elevation. This is also the back of a marine terrace that is a well-defined topographic feature of the south coast of the Perachora Peninsula and represents uniform uplift of marine terraces on the footwall of an active off-shore fault trending parallel to the coastline (Leeder et al., 2003; Turner et al., 2010). Well-preserved coral samples from other locations beneath the same marine terrace are reliably dated to MIS 5e (Leeder et al., 2005 and references therein). At Agriliou Bay a higher terrace (to ~70 m) is reliably dated to MIS 7, and the lower terrace (~12 m) to MIS 5a/c; thus the range in ages correlating with MIS 5e is attributed to open-system behaviour of these poorly-preserved corals (Turner et al., 2010). *In-situ Pecten* from

this marine sequence were sampled from an exposure ~8 m above modern sea level, and overlain by ~4 m semi-lithified near-shore sediments. This location is incised by a river channel which cuts down through the uplifted marine terraces.

- The corals and associated *Pecten* shells Gr 10 and 11 were collected from 10-12 m elevation at Goat Point, a rubbly terrace of reworked bioherms capping marly sediments with marine fossils. The coral associated with Gr 10 was dated between 142.7 +4.6/-4.7 ka and 140.3+4.5/-4.6 ka, which corresponds to late MIS 6/early MIS 5e, and independently interpreted as MIS 5e age on stratigraphic relationships by Portman et al., (2005). This terrace can be correlated along the coastline with the ~30 m MIS 5e marine terrace beneath which Gr 11 was also sampled.

MIS 5a/c: The corals and associated *Pecten* shell Gr 12 were collected from a 12 m terrace at Australia Gorge, the sample elevation being ~ 6 m above sea level. Coral samples were U-Th dated to 81.9 +1.9/-1.9 ka and thus correlate with MIS 5a/c.

Test shell Gr9 (MIS 5, substage unknown): The corals and associated *Pecten* shell Gr 9 were collected from 6 m elevation at Flagnoro Bay, and whilst the corals yielded a MIS 5a/c age (90.4 +2.3/-2.3 ka; dated by the Open University Uranium-Series Facility (OOUSF); University of East Anglia (UEA) unpublished data), coral from a similar elevation, 110 m to the east, was dated to MIS 5e by Roberts et al. (2009; their sample S2, Table 1).

2.3 Sample preparation

A. Fossil shells: All sub-fossil *Pecten* shells were sampled on the rim to obtain a fragment ~2x5 cm. The shell fragments were first cleaned with a rotary drill to remove any adhering sediment and then sonicated several times in ultrapure water. Dry fragments were crushed to a fine powder (~500 μm), as this size has been found to be optimal for other biominerals investigated for IcPD in the NEaar laboratory (Penkman et al., 2008; Demarchi et al., 2013b), and two samples taken from each powdered fragment. Following the results of the bleaching experiment (Section 3.1), a 48-h bleaching step was used to isolate the intra-crystalline fraction.

B. Modern shells for laboratory experiments: Five modern *Pecten* sp. shells, bought at a local market in York (UK), were used for bleaching and high-temperature experiments, according to the standard protocol of the NEaar laboratory, described in Penkman et al. (2008). The microstructure of the valves of scallop shells consists of two layers of foliated calcite (Carter, 1989). Here we sampled the rim of each shell in order to select the outer layer only and thus minimise intra-shell variability. The rim of each shell was sampled, cleaned by drilling the periostracum off and sonicating in ultrapure water, and then powdered to ~500 μm particle size.

C. Bleaching experiments: Sequential bleaching experiments were performed by soaking the bulked powders in NaOCl (12% w/v, 50 μL per mg) for various times, in order to assess the effectiveness of bleaching on *Pecten* and determine the optimum time necessary to isolate the intra-crystalline fraction (Penkman et al., 2008; Orem and Kaufman, 2011; Crisp et al., 2013; Demarchi et al., 2013b,c; Tomiak et al., 2013). Five different exposure times to the oxidizing agent were tested (0, 24, 48, 72, 168 h) and three laboratory replicates prepared for each time point (Supplementary Information).

D. High-temperature (kinetic) experiments: For high-temperature experiments, approximately 20 mg of dry shell powders (bleached for 48 hours) were weighed into individual sterile glass ampoules and 300 µL of ultrapure water added to each sample. According to the NEaar laboratory protocols, no silica glass was added, as the experiments were performed in water and sealed under ambient atmospheric conditions. Three laboratory replicates were prepared for each time point at each temperature. The ampoules were flame-sealed and placed in an oven at 140˚C, 110˚C or 80˚C for various times (Table 2). Water blanks were prepared for selected time points and heated alongside the powder samples (Supplementary Information). After heating, the supernatant water was removed, and the powders left to air-dry before undergoing further preparation for FAA and THAA analysis (section 2.4).

E. Leaching experiments: For this subset of experiments, the amino acid concentrations detected both in the heated powders and the waters were considered. We compared 48-h bleached and unbleached powders heated at 140°C for 1 hour and 24 hours.

2.4 Analysis of intra-crystalline amino acids

Each powdered *Pecten* sample was separated into two further subsamples for the analysis of the FAA and THAA fractions. The word "sample" here represents: a) bleached powder of modern shells heated at a certain temperature for a certain time (three laboratory replicates per time point); b) bleached powdered fragments taken from the rim of fossil shell individuals (two per specimen).

FAA and THAA subsamples were respectively demineralised in 2 M HCl or hydrolysed in 7 M HCl (at 110 °C for 24 hours in nitrogen-flushed vials) and then dried in a centrifugal evaporator according to the standard protocol (Penkman et al., 2008). For leaching experiments, the amino acid concentrations in the water (THAAw) were obtained by removing a 100 µL aliquot of the supernatant water and hydrolysing the peptide bonds in 6M HCl (20 µL per mg/equivalent; Penkman et al., 2008), at 110°C for 24 hours.

Overall, we obtained:

- For bleaching and kinetic experiments: one FAA/THAA pair per laboratory replicate, for a total of three FAA and three THAA subsamples per time point;
- For leaching experiments: one THAAw subsample per laboratory replicate, for a total of three THAAw subsamples per time point;
- For fossil *Pecten*, 2 THAA and 2 FAA subsamples per shell specimen.

For the analysis of chiral amino acids by reverse-phase liquid chromatography (RP-HPLC), following a modified method of Kaufman and Manley (1998), the extracted amino acids were rehydrated with 10 µL rehydration fluid (containing the non-protein amino acid L-homo-arginine as the internal standard) per mg of original bleached shell powder. Each rehydrated vial was typically analysed once by RP-HPLC; however, a subset of the fossil shell samples was analysed twice (Supplementary Information). Standard solutions of known D/L value and concentrations and procedural blanks were routinely prepared and interspersed in each analytical run. Background levels were calculated by averaging the amino acid concentration detected in the blanks and by calculating the limit of detection LOD = Total $[THAA]_{blanks}$ x 3.

During preparative hydrolysis, both asparagine and glutamine undergo rapid irreversible deamination to aspartic acid and glutamic acid, respectively (Hill, 1965), and they are reported together as Asx and Glx. The amino acids reported and discussed in this study are those with optimal chromatographic resolution: Asx, Glx, Gly, Ser, Ala, Val. The amino acids Phe, Leu, Ile are discussed where appropriate, but due to a lack of baseline resolution, they provide only qualitative data.

3. Results and Discussion

3.1 Characterising the intra-crystalline fraction in Pecten

Here we compare the relative amino acid composition of modern *Pecten* shells from our study with that determined in sequenced matrix proteins of scallop shells (*Mizuhopecten yessoensis* - *Patinopecten yessoensis*): MSP-1 and SP-S (Sarashina and Endo, 1998, 2001; Hasegawa and Uchiyama, 2005) and the two nacrein-like proteins P1 and P2 (Norizuki and Samata, 2008), which act as a negative regulator of calcification in the shells of molluscs. Cys, Lys, Pro and Trp are not routinely quantified with our RP-HPLC method; Tyr is observed but was not quantified for this study, while Met degrades (oxidises) during preparation, therefore we adjusted the theoretical composition of the matrix proteins accordingly. We note that the composition of unbleached *Pecten* (Fig. 3A) is similar to that of MSP-1 and SP-S, with high relative proportions of Ser, Gly, Asx, Ala and Glx (Fig. 3C and 3D). The percentage values differ (e.g. Asx represent 24% and 28% of the composition of MSP-1 and SP-S, and 32% in *Pecten*), possibly because the measure of bulk amino acids from a fraction of proteins of unknown sequence might not be the same as the theoretical composition of known sequences; however, the overall proportion of Asx, Ser and Gly are broadly equivalent in MSP-1, SP-S, and unbleached *Pecten.* On the contrary, the nacrein-like proteins P1 and P2 have higher relative levels of the acidic amino acids Asx and Glx compared to Ala, Gly and Ser (Figs 3E and 3F).

Bleaching has a significant effect on the matrix proteins retained in *Pecten*: after 48 h exposure to NaOCl, only 6% of the original THAA are retained (Fig. 4A), a value that remains stable after prolonged bleaching. Interestingly, the relative percentage of Ser and Gly decreased in bleached shells compared to unbleached powders, although still dominated by Asx, Glx, Ser and Gly (Fig. 3B). This may indicate that Asx and Glx-rich nacrein-like proteins dominate the intra-crystalline pool, while Ser and Gly-rich MSP and SP proteins are mainly inter-crystalline. The comparison suggests that two very different pools of proteins characterise the intra- and inter-crystalline environments in *Pecten*. The Asx- and Glx-rich fraction may even offer superior performance as a substrate for geochronology over Quaternary timescales rather than the Ser-rich pool, as Ser diagenesis is complicated by decomposition and a reversal in D/L values (section 3.3). Further, the knowledge that nacrein-like proteins are associated with the (operational) intra-crystalline fraction supports the hypothesis that these play an important role in biomineralisation itself (Norizuki and Samata, 2008).

The extent of bleach-induced racemization is negligible between 24 and 72 hours (< 0.03 D/L units for Asx; Figure 4B and Supplementary Information), although after 72 h bleaching a further increase is observed for Asx and Ser, thus suggesting that longer bleaching times may compromise the integrity of the carbonate phase. Therefore we chose a 48-h bleaching step (standard for the NEaar laboratory) for all further analyses on *Pecten*. The percentage of

amino acids removed by 48-h bleaching in *Pecten* is ~95%, higher than for other bleached marine molluscs (e.g. 85-90% in *Patella*, 30% in *Glycymeris* (Demarchi et al., 2013b, 2015; Ortiz et al., 2015)). The average value of total THAA concentration in 48-h bleached *Pecten* powders was ~2.6 nmol/mg, similar to 48-h bleached *Patella* (~2.8 nmol/mg) and higher than bleached *Glycymeris* (~1.5 nmol/mg) (Demarchi et al., 2013b, 2015).

These results provide evidence for the presence of an operationally defined intra-crystalline fraction of proteins that can be isolated by a 48-h bleaching step, similar to previous observations for other carbonate biominerals (Penkman et al., 2008; Hendy et al., 2012; Demarchi et al., 2013b).

3.2 Leaching experiments: test of closed-system behaviour

Closed-system tests are used to assess the stability of the intra-crystalline fraction upon conditions of continuous leaching in the laboratory, accelerated by high temperatures. In the NEaar laboratory, heating experiments are typically conducted on a homogenised "bulk" sample of bleached powders from modern shells (section 2.3) in order to determine the extent of any leaching from the system. However, it is important to note that heating bleached powders might not accurately mimic the conditions in the natural environment. Therefore, while our approach provides a useful proxy to compare the behaviour of unbleached and bleached shell powders, we stress that results presented on bleached shells here (and in other studies, e.g. Penkman et al., 2008; Demarchi et al., 2105) may not be comparable to data obtained by other authors on unbleached material (e.g. Hare and Mitterer, 1969; Miller et al 2000; Kaufman, 2006; Ortiz et al., 2015).

Results on bleached and unbleached *Pecten* powders show that significant leaching of amino acids into the water occurred for the unbleached material (21% of the overall amino acid concentration after 24 h heating). The amino acid concentration detected in the water from the bleached samples was lower than background levels (LOD) (Fig. 5A) and represents ~0. 4% after 24 h heating (Table 3). While this may indicate that the system (heated bleached shells) is not perfectly closed, the high levels of leaching from unbleached shells support the hypothesis that the isolation of the intra-crystalline fraction in fossil *Pecten* may be important for geochronological studies.

It is important to note that the isolation of the intra-crystalline fraction results in major differences in racemization values. Figs. 5B-C-D-E display THAA D/L values detected in bleached and unbleached shell powders heated for 1 and 24 hours at 140°C: despite the limited dataset (two time-points only), the intra-crystalline fraction is significantly more racemized (up to 0.5 D/L units difference) than its unbleached ("whole-shell"), counterpart, indicating that the pathways of early diagenesis in bleached and unbleached shells diverge. Therefore, patterns of IcPD (i.e. on bleached shells – section 3.3) will not necessarily mimic patterns observed when unbleached shells are analysed.

3.3 Patterns of IcPD at high and low temperatures

The results of bleaching and leaching experiments show that bleached modern *Pecten* samples yield a fraction of intra-crystalline amino acids which is not leached under conditions of prolonged artificial diagenesis (Figs 4 and 5A). Furthermore, high-temperature experiments demonstrate that the patterns of hydrolysis, decomposition and racemization are predictable: Fig. 6 shows that the %FAA (i.e.: [FAA]/[THAA]x100) and D/L values increase

steadily with heating time, although %FAA decrease for the last time-point (120 h), presumably due to decomposition (140 °C data in Fig. 6; 110 °C and 80 °C data in Supplementary Information). A well-known exception to the increase in THAA D/Ls over time is represented by Ser, which displays a reversal (i.e. in *Pecten* D/Ls increase to ~0.5 and then decrease). In addition [Ser] values fall below the LOD in bleached *Pecten* heated at 140°C for 2 hours. Nonetheless, the initial diagenesis of Ser and the patterns of degradation of the fast-racemizing Asx can be useful as a geochronometer for shorter timescales (e.g. Demarchi et al., 2011; Hendy et al., 2012), while slow-racemizing residues (Glx, Val) could be useful for older material. The very high levels of racemization of Ala (usually a medium-rate racemizer), may be affected by the rapid decomposition of Ser, thus resulting in higher-than-expected DL ratios: in the 140°C experiment, THAA Ser concentrations fall from ~400 to ~100 pmol/mg between unheated and 1-h heated bleached samples. In the same time interval, THAA Ala concentrations increase by ~60 pmol/mg, therefore accounting for less than one third of the loss in THAA Ser. The increase in THAA Ala D/Ls may also be due to the positions of Ala in the protein chain (e.g. if a significant proportion was next to a rapidly-hydrolysed peptide bond, resulting in enhanced racemization of Ala in an N-terminal position (Mitterer and Kriausakul, 1984)).

The predictable patterns of diagenesis displayed by bleached *Pecten* heated at high temperatures suggest that *Pecten* has very good potential as a substrate for IcPD geochronology. However, diagenesis at high temperature may differ significantly from the breakdown pathways occurring at the normal burial temperature, as we have reported for a range of biominerals, including marine mollusc shells and corals (Demarchi et al., 2013c; Tomiak et al., 2013). “Natural” diagenesis of bleached subfossil *Pecten* appears to behave in a way that resembles closely the high-temperature patterns with regards to racemization (FAA D/L vs THAA D/L plots, Figs 7A, 7C, 7E, 7G). On the contrary, %FAA vs THAA D/L plots (Figs 7B, 7D, 7F, 7H) show that, for the same THAA D/L, bleached shell powders heated at high temperatures yielded lower %FAA values than fossil bleached shells. We observed a similar pattern in bleached *Patella* (Demarchi et al., 2013c) and interpreted it as indicating that the activation energies for peptide bond hydrolysis were lower than those for racemization, which implies that at lower temperatures hydrolysis will be more facile than racemization. We cautiously suggest a similar interpretation here, which however will require further testing on a more extensive set of high-temperature experiments on both unbleached and bleached *Pecten*. We also note that the higher variability displayed by fossil data may be partly due to the localised burial/thermal environment of each shell, but also natural inter-shell variability in protein composition in individual shells, compared to the homogenised powders used for kinetic experiments.

The variability of the fossil data is lower in THAA vs FAA D/Ls plots than in THAA D/Ls vs %FAA plots and the trajectory of co-variance is coherent (from less degraded to most degraded), thus indicating good closed-system behaviour. The only exception is represented by FAA D/L values for Glx, but this is likely to be due to the preferential formation of FAA Glx as a lactam (e.g. Walton, 1998), which cannot be detected with our analytical method.

However, as kinetic experiments were conducted on powders bleached pre-heating rather than post-heating, the patterns observed here might not mimic the pathways of diagenesis occurring in the natural environment. An example of these dissimilarities is highlighted by the difference in total amino acid concentrations recovered from fossil and modern shells.

The sum of Asx, Glx, Ser, Ala, Gly and Val THAA concentrations found in bleached fossil shells of MIS 5 age (~3.5 nmol/mg, ±12%) is higher than that found in bleached modern shell powders (~2.6 nmol/mg). MIS 7 and MIS 9 shells also yielded slightly higher values (~2.8 and 3.2 nmol/mg, respectively), while the concentrations in the MIS 11 specimen were lower (~1.8 nmol/mg) (Supplementary Information). Higher-than-expected concentrations in the fossils indicate that diagenesis may result in matrix-protein interactions which may not be disrupted by bleaching, resulting in the isolation of a different fraction of organics. This does not preclude that the fraction isolated from fossil shells appear to behave as a closed system: the covariance of FAA vs THAA D/L values (Fig. 7) shows that open-system behaviour does not seem to affect the shells analysed as part of this study.

These results indicate that whilst *Pecten* is a shell taxon that should be targeted for IcPD dating, the patterns of diagenesis are (unsurprisingly) as complex as those found in other biominerals, particularly regarding the temperature sensitivity of the different breakdown reactions. We have not used the high temperature kinetic data to extrapolate reaction rate parameters to burial temperatures, but larger fossil datasets should enable kinetic modelling in future studies.

3.4 Using bleached Pecten *as a geochronological indicator for the Quaternary of the Mediterranean*

Calibration of the extent of IcPD with independent numerical dating methods is especially important over Quaternary timescales encompassing multiple glacial-interglacial cycles (see Wehmiller et al., 2012) and in sedimentary deposits in mid-latitude areas with strong temperature gradients, such as the Mediterranean. In this study, we tested the feasibility of using bleached *Pecten* for aminostratigraphy, using nine bleached *Pecten* shells from seven Pleistocene raised beaches, located in the area of the Corinth Gulf, dated by U-Th and ranging from 81 ka to > 350 ka (Table 1). The aim of this preliminary work was to assess whether the protein diagenesis in the intra-crystalline fraction of subfossil *Pecten* showed a consistent pattern with age, the potential levels of resolution and to establish the temporal span of the technique on this substrate in this geographic area.

Applying statistical analyses to AAR data is complicated by the fact that D/L values have upper and lower boundaries, and the small sample sizes in this study limit the confidence in the statistical results, but the data were interrogated using ANOVA and Bonferroni-Holm tests. Here, the number of shell samples for MIS5 is much higher than for other stages; the results of the Bonferroni-Holm test (Supplementary Information) should therefore be viewed with caution. Despite these limitations we report the results of the statistical tests to provide preliminary indication on the suitability of *Pecten* as a substrate for IcPD for age estimation.

Asx THAA D/Ls (Fig. 8A, note scale) are still relatively far from equilibrium (D/L=1) and are able to provide well-resolved age differences between shells belonging to different MISs: significant differences are found between MIS 5 / MIS 9 and MIS 5 / MIS 11, but not between MIS 5 / MIS 7, MIS 7 / MIS 9 or MIS 9 / MIS 11, although a general increase of D/L values with age can be observed (Fig. 8A). Asx MIS 11 values (Gr 5) are lower than expected (similar to MIS 7 values), and this has an impact on the age resolution. Asx is the only amino acid displaying this pattern for Gr5, but concentrations for this shell are lower than for other shells analysed in this study (Supplementary Information); we therefore hypothesise that this result might be due to decomposition of the highly-racemized FAA Asx

fraction., Any within-MIS 5 differences could not be resolved, and the test shell Gr9 did not show significant differences with either MIS 5e or MIS 5a/c (or MIS 7) shells.

The amino acid Ala (Fig. 8E), usually a medium-rate racemizer, displays D/L values between 0.45-0.85, higher than those of the fast racemizer Asx, as already shown by the high-temperature dataset (Fig. 6). The rapid decomposition of Ser into Ala (observed in the kinetic experiments) indicates that the patterns observed may reflect the interplay of decomposition and racemization of both amino acids. Ala THAA D/Ls for MIS 5 are significantly different from MISs 7, 9, 11 but MISs 7, 9 and 11 cannot be resolved (Bonferroni-Holm results in Supplementary Information). The level of variability within MIS 5e, although not exceptionally high (CVs ~9%) is largely due to Gr13, which falls at slightly lower D/L values than other MIS 5e shells. THAA [Ser]/[Ala] values (Fig. 8D) might reflect directly the decomposition of Ser into Ala, but the only statistically significant difference is found between Gr9 and MIS 7 shells. These results therefore strengthen the conclusion that Gr9 is MIS 5 in age, although the sub-stage within MIS 5 cannot be better resolved.

Slow-racemizing amino acids (Glx and Val; Figs 8B and 8C) should represent the best indicators of diagenesis over Pleistocene timescales; however, in bleached *Pecten* both amino acids display high D/Ls and therefore this limits their usefulness for older shells (here, MIS 7-11). Glx THAA D/Ls are able to resolve age differences between shells of MIS 5 and MISs 7/9/11 age; shells Gr9 (test sample) and Gr12 (MIS 5a/c) are also significantly different, thus supporting an attribution of Gr9 to an earlier substage within MIS 5. Val THAA D/Ls perform slightly worse than Glx D/Ls over younger timescales, with both MIS 5 values indistinguishable from MIS 7 D/Ls, although significantly lower than MIS 9 and MIS 11.

4. Conclusions

The extent of protein degradation in modern and sub-fossil *Pecten* shells was investigated through the concurrent use of the closed system approach of Penkman et al. (2008) and the analysis of multiple amino acids (Kaufman and Manley, 1998). When tested against the raised beaches located in the area of the Corinth Gulf, the IcPD in subfossil *Pecten* showed a consistent diagenetic pattern with age. Our results showed that:

- A 48-h bleaching step is effective in isolating the intra-crystalline fraction of proteins shells of the modern marine mollusc *Pecten* (Fig. 4), similar to that observed for other mollusc shells (Sykes et al., 1995; Penkman et al., 2008; Demarchi et al., 2013b).
- The intra-crystalline proteins are likely to belong to the group of nacrein-like proteins on the basis of their amino acid composition (Fig. 3).
- The minimal loss of amino acids from the bleached shell during high temperature (leaching) experiments supports the evidence for the system's integrity (Fig. 5), and we suggest that the intra-crystalline fraction of *Pecten* shells better approximated a closed system than the unbleached fraction.
- *Pecten* shows good potential for retaining an effectively closed system of proteins over geological timescales (Fig. 7), therefore it can be identified as a useful substrate for future IcPD geochronological studies.
- The diagenetic patterns followed by fossil shells and modern shells heated at high temperature were similar for the amino acids Asx, Glx, Ala and Val, but only when the co-variance of FAA vs THAA D/Ls were considered, whilst plots of THAA D/Ls

vs %FAA showed significant differences between the reactions at high and low temperatures (Figs 6, 7).

- The small sample sizes in this preliminary study preclude a full assessment of the aminostratigaphic power of *Pecten* IcPD, but this pilot study shows that the resolution of IcPD data on *Pecten* for the Gulf of Corinth is potentially sufficient to distinguish between shells of MIS 5 age from older material. It was not possible to differentiate the single specimens analysed from MIS 7, 9 and 11 deposits in this study, although we observed a coherent increase in Ala, Glx and Val D/L values and decrease in [Ser]/[Ala] with age between MIS 7 and MIS 11.
- Val and Ala D/L values approach equilibrium for MIS 11 shells and this results in loss of resolution for shells older than 350 ka. However, Glx is further from equilibrium for shells of this age, and may allow the limits of the technique to be extended beyond MIS 11 in the Mediterranean area.
- The analysis of a larger number of fossil shells in future studies will allow the assessment of the within-MIS natural variability and the level of resolution to be refined.

Acknowledgments

This work was funded by the EU Reintegration grant (PERG07-GA-2010-268429, FP 7), project "mAARiTIME", the Erasmus Placement Programme and the Leverhulme Trust. Matthew Collins is thanked for useful discussion and support. We are grateful to Darrell Kaufman (Editor), José Eugenio Ortiz and an anonymous reviewer for their insightful comments on the manuscript.

References

Belluomini, G., Delitala, L., 1988. Amino acid racemization dating of Quaternary deposits of central and Southern Italy. Org. Geochem. 13, 735–740.

Carter, J.G., 1989. Skeletal biomineralization: patterns, processes and evolutionary trends. Short Courses in Geology, vol. 5. New York, Van Nostrand Reinhold, pp. 1-399.

Collier, R.E.L., 1990. Eustatic and tectonic controls upon Quaternary coastal sedimentation in the Corinth Basin, Greece. J. Geol. Soc. London. 147, 301–314.

Crisp, M., Demarchi, B., Collins, M., Morgan-Williams, M., Pilgrim, E., Penkman, K., 2013. Isolation of the intra-crystalline proteins and kinetic studies in Struthio camelus (ostrich) eggshell for amino acid geochronology. Quat. Geochronol. 16, 110–128.

Demarchi, B., Collins, M.J., Bergstrom, E., Dowle, A., Penkman, K.E.H., Thomas-Oates, J., Wilson, J., 2013a. New experimental evidence for in-chain amino acid racemization of serine in a model peptide. Anal. Chem. 85 (12), 5835–5842.

Demarchi, B., Rogers, K., Fa, D.A., Finlayson, C.J., Milner, N., Penkman, K.E.H., 2013b. Intra-crystalline protein diagenesis (IcPD) in *Patella vulgata*. Part I: Isolation and testing of the closed system. Quat. Geochronol. 16, 144–157.

Demarchi, B., Collins, M.J., Tomiak, P.J., Davies, B.J., Penkman, K.E.H., 2013c. Intra-crystalline protein diagenesis (IcPD) in *Patella vulgata*. Part II: Breakdown and temperature sensitivity. Quat. Geochronol. 16, 158–172.

Demarchi, B., Clements, E., Coltorti, M., van de Locht, R., Kröger, R., Penkman, K., Rose, J., 2015. Testing the effect of bleaching on the bivalve *Glycymeris*: A case study of amino acid geochronology on key Mediterranean raised beach deposits. Quat. Geochron. 25, 49-65.

Dia, A., Cohen, A., O'Nions, R., Jackson, J., 1997. Rates of uplift investigated through 230Th dating in the Gulf of Corinth (Greece). Chem. Geol. 138, 171–184.

Hare, P.E., Mitterer, R.M, 1969. Laboratory Simulation of amino acid diagenesis in fossils. Carnegie Institute of Washington Yearbook 67, 205-8.

Hasegawa, Y., Uchiyama, K., 2005. cDNA clonings of shell matrix proteins from scallop shell. Fish. Sci. 71, 1174–1178.

Hearty, P.J., 1986. An inventory of last interglacial (s.l.) age deposits from the Mediterranean basin: a study in isoleucine epimerization and U-Th dating. Zeitschrift fur Geomorphol. Supplement, 62, 51–69.

Hearty, P.J., 1987. New data on the pleistocene of Mallorca. Quat. Sci. Rev. 6, 245–257.

Hearty, P.J., Miller, G.H., Stearns, C.E., Szabo, B.J., 1986. Aminostratigraphy of Quaternary shorelines in the Mediterranean basin. Geol. Soc. Am. Bull. 97, 850–858.

Hearty, P.J., Dal Pra, G., 1992. The Age and Stratigraphy of Middle Pleistocene and Younger Deposits Along the Gulf of Taranto (Southeast Italy). J. Coast. Res. 8, 882–905.

Hendy, E.J., Tomiak, P.J., Collins, M.J., Hellstrom, J., Tudhope, A.W., Lough, J.M., Penkman, K.E.H., 2012. Assessing amino acid racemization variability in coral intra-crystalline protein for geochronological applications. Geochim. Cosmochim. Acta 86, 338–353.

Hill, R.L., 1965. Hydrolysis of Proteins. Adv. Protein Chem. 20, 37–107.

Kaufman, D.S., 2006. Temperature sensitivity of aspartic and glutamic acid racemization in the foraminifera *Pulleniatina*. Quat. Geochron. 1, 188-207.

Kaufman, D.S., Manley, W.F., 1998. A new procedure for determining dl amino acid ratios in fossils using reverse phase liquid chromatography. Quat. Sci. Rev. 17, 987–1000.

Leeder, M.R., McNeill, L.C., Collier, R.E.Ll, Portman, P., Rowe, P.J., Andrews, J.E., Gawthorpe, R.L., 2003. Corinth rift margin uplift: New evidence from Late Quaternary marine shorelines. Geophys. Res. Lett. 30, 1611.

Leeder, M.R., Portman, C., Andrews, J.E., Collier, R.E.L., Finch, E., Gawthorpe, R.L., McNeill, L.C., Pérez-Arlucea, M., Rowe, P., 2005. Normal faulting and crustal deformation, Alkyonides Gulf and Perachora peninsula, eastern Gulf of Corinth rift, Greece. J. Geol. Soc. 162, 549–561.

Miller, G.H., Hare, P.E., 1980. Amino acid geochronology: integrity of the carbonate matrix and potential of molluscan fossils. In: Biogeochemistry of amino acids. New York, Wiley, pp. 415-443.

Miller, G.H., Hart, C.P., Roark, E.B., Johnson, B.J., 2000. Isoleucine epimerization in eggshells of the flightless Australian birds *Genyornis* and *Dromaius*. In: *Perspectives in Amino Acid and Protein Geochemistry*, ed. G.A. Goodfriend, M.J. Collins, M.L. Fogel, S.A. Macko, J.F. Wehmiller. New York, Oxford University Press, pp. 161-81.

Mitterer, R.M., Kriausakul, N., 1984. Comparison of rates and degrees of isoleucine epimerization in dipeptides and tripeptides. Org. Geochem. 7, 91–98.

Morewood, N.C., Roberts, G.P., 1999. Lateral propagation of the surface trace of the South Alkyonides normal fault segment, central Greece: its impact on models of fault growth and displacement–length relationships. J. Struct. Geol. 21, 635–652.

Murray-Wallace, C.V., Farland, M.A., Roy, P.S., Sollar, A., 1996. Unravelling patterns of reworking in lowstand shelf deposits using amino acid racemization and radiocarbon dating. Quat. Sci. Rev. 15, 685–697.

Murray-Wallace, C.V., Farland, M.A., Roy, P.S., 2005. Further amino acid racemization evidence for glacial age, multiple lowstand deposition on the New South Wales outer continental shelf, southeastern Australia. Mar. Geol. 214, 235–250.

Norizuki, M., Samata, T., 2008. Distribution and Function of the Nacrein-Related Proteins Inferred from Structural Analysis. Mar. Biotechnol. 10, 234–241.

Orem, C.A., Kaufman, D.S., 2011. Effects of basic pH on amino acid racemization and leaching in freshwater mollusk shell. Quat. Geochronol. 6, 233–245.

Ortiz, J.E., Gutiérrez-Zugasti, I., Torres, T., González-Morales, M., Sánchez-Palencia, Y., 2015. Protein diagenesis in *Patella* shells: Implications for amino acid racemisation dating. Quat. Geochronol. 27, 105-118.

Penkman, K.E.H., Kaufman, D.S., Maddy, D., Collins, M.J., 2008. Closed-system behaviour of the intra-crystalline fraction of amino acids in mollusc shells. Quat. Geochronol. 3, 2–25.

Penkman, K.E.H., Preece, R.C., Bridgland, D.R., Keen, D.H., Meijer, T., Parfitt, S.A., White, T.S., Collins, M.J. 2013. An aminostratigraphy for the British Quaternary based on *Bithynia* opercula. Quat. Sci. Rev. 61, 111-134.

Portman, C., Andrews, J.E., Rowe, P.J., Leeder, M.R., Hoogewerff, J., 2005. Submarine-spring controlled calcification and growth of large *Rivularia* bioherms: Late Pleistocene (MIS 5e), Gulf of Corinth, Greece. Sedimentology 52, 441-465.

Roberts, G.P.; Houghton, S.L.; Underwood, C.; Papnikolaou, I.; Cowie, P.A.; van Calsteren, P.; Wigley, T.; Cooper, F.J.; McArthur, J.M., 2009 Localization of Quaternary slip rates in an active rift in 10(5) years: an example from central Greece constrained by U-234-Th-230 coral dates from uplifted paleoshorelines. J. Geophys. Res.: Solid Earth 114, B10406.

Sarashina, I., Endo, K., 1998. Primary structure of a soluble matrix protein of scallop shell; implications for calcium carbonate biomineralization. Am. Mineral. 83, 1510–1515.

Sarashina, I., Endo, K., 2001. The Complete Primary Structure of Molluscan Shell Protein 1 (MSP-1), an Acidic Glycoprotein in the Shell Matrix of the Scallop *Patinopecten yessoensis*. Mar. Biotechnol. 3, 362–369.

Sykes, G.A., Collins, M.J., Walton, D.I., 1995. The significance of a geochemically isolated intracrystalline organic fraction within biominerals. Org. Geochem. 23, 1059–1065.

Tomiak, P.J., Penkman, K.E.H., Hendy, E.J., Demarchi, B., Murrells, S., Davis, S.A., McCullagh, P., Collins, M.J., 2013. Testing the limitations of artificial protein degradation kinetics using known-age massive Porites coral skeletons. Quat. Geochronol. 16, 87–109.

Torres, T., Llamas, J., Canoira, L., Coello, F. J., Garcia-Alonso, P., Ortiz, J. E., 2000. Aminostratigraphy of two Pleistocene marine sequences from the Mediterranean coast of Spain: Cabo de Huertas (Alicante) and Garrucha (Almería). Perspectives in Amino Acids and Protein Geochemistry, 263-278.

Torres, T., Ortiz, J. E., Arribas, I., Delgado, A., Julia, R., & Martín-Rubí, J. A., 2010. Geochemistry of *Persististrombus latus* Gmelin from the Pleistocene Iberian Mediterranean realm. Lethaia, 43(2), 149-163.

Turner, J., Leeder, M., Andrews, J., Rowe, P., Van Calsteren, P., Thomas, L., 2010. Testing rival tectonic uplift models for the Lechaion Gulf in the Gulf of Corinth rift. J. Geol. Soc. 167, 1237-1250.

Vita-Finzi, C., King, G.C.P., 1985. The Seismicity, Geomorphology and Structural Evolution of the Corinth Area of Greece. Philos. Trans. R. Soc. London. Ser. A, Math. Phys. Sci. 314, 379–407.

Walton, D, 1998. Degradation of intracrystalline proteins and amino acids in fossil brachiopods. Org. Geochem. 28.6, 389-410.

Wehmiller, J.F., 1977. Amino acid studies of the Del Mar, California, midden site: apparent rate constants, ground temperature models, and chronological implications. Earth and Planetary Sci. Lett., 37(2), 184-196.

Wehmiller, J.F., 1982. A review of amino acid racemization studies in Quaternary mollusks: stratigraphic and chronologic applications in coastal and interglacial sites, Pacific and Atlantic coasts, United States, United Kingdom, Baffin Island, and tropical islands. Quat. Sci. Rev., 1(2), 83-120.

Wehmiller, J.F., Harris, W.B., Boutin, B.S., Farrell, K.M., 2012. Calibration of amino acid racemization (AAR) kinetics in United States mid-Atlantic Coastal Plain Quaternary mollusks using $^{87}Sr/^{86}Sr$ analyses: Evaluation of kinetic models and estimation of regional Late Pleistocene temperature history. Quat. Geochronol. 7, 21-36.

Tables

Table 1: Details of fossil *Pecten* sp. shells from raised beach deposits of the Corinth Gulf, Greece, analysed for IcPD dating. Numerical ages (ka) were obtained by U/Th dating (TIMS) on *in-situ* coral samples. [1](Collier, 1990); [2] (Leeder et al., 2003); [3] (UEA samples dated by OUUSF); [4](Leeder et al., 2005); [5](Dia et al., 1997).

Sample name	Site name	Control U/Th Age (ka)	Marine Isotope Stages	Subsamples per shell (n)	NEaar numbers
Gr5	Corinth Canal	>350	[1]MIS 11	2	6856/6857
Gr6	Corinth Canal	311.8 +33.4/-25.8	[1]MIS 9	2	6858/6859
Gr7	Corinth Canal	205.2 +13.0/-11.7	[1]MIS 7	2	6860/6861
Gr8	Makrugoaz Ridge	108.5±0.7 to 134.0±32.0	[2]MIS 5e	2	6862/6863
Gr9	Flagnoro Bay	90.4+2.3/-2.3	[3]MIS 5	2	6864/6865
Gr10	Goat Point	142.6+4.6/-4.7 - 140.3+4.5/-4.6	[3]MIS 5e	2	6866/6867
Gr11	Goat Point	stratigraphic correlation	MIS 5e	2	6868/6869
Gr12	Australia Gorge	81.9+1.9/-1.9	MIS 5a/c	2	6870/6871
Gr13	New Corinth Terrace	132.0+8.2/-7.5	[4]MIS 5e	2	6872/6873
Gr14	Agriliou Bay	Between 123.1±0.7 and 120.5±0.9	[5]MIS 5e	1	6874
Gr15	Agriliou Bay	Between 123.1±0.7 and 120.5±0.9	[5]MIS 5e	2	6876/6877

Table 2. Temperatures and time points used for kinetic experiments on bleached *Pecten* shell. * unbleached samples were also prepared for these time points. Water samples were analysed for bleached and unbleached powers heated for 1 and 24 hours at 140°C.

Temperature (°C)	**Time points (h)**						
140	1*	2	4	8	24*	48	120
110	24	120	240	481	865	1200	
80	24	99	120	720	1200		

Table 3. % loss of amino acids from unbleached and bleached *Pecten* powders after 24 h of heating at 140 °C (n = number of samples). Total concentrations were calculated using Asx, Glx, Ser, Gly, Ala and Val. The average blank total concentration (147 pmol/mg) was subtracted from all values.

Loss of amino acids after 24 h heating at 140 °C	**Unbleached**	**Bleached**
Initial [total] THAA concentration in shell, unheated (pmol/mg) (n = 3)	52757	2366
[total] THAA after heating (pmol/mg) for 24 h	5850	1580
[total] THAAw in water, heated (pmol/mg equiv.)	11059	10
Overall loss in shell (%)	89%	33%
Loss into water, by leaching (%)	21%	0.4%
Loss by decomposition (%)	68%	33%

Figure Legends

Figure 1. Sampling location in the eastern Mediterranean (A): the Perachora Peninsula and Corinth Canal in the Gulf of Corinth, Central Greece (B). C: Location of *Pecten* and coral samples from around the uplifted coastline.

Figure 2. During each marine highstand the marine sediments aggrade and prograde, forming a sequence of sediments associated with each highstand which typically coarsen up-section, from off-shore marine to shallow marine to beach facies (A). As the region has been uplifting since at least MIS 11, these sediments are raised above sea level with well-preserved fossil material. The white square in Fig. 2A (enlarged in Fig. 2B) shows the sample location for Gr 5 (MIS 11). C: *Pecten jacobaeus*.

Figure 3. THAA composition of unbleached (A) and 48-h bleached (B) *Pecten* shells. Sequenced matrix proteins of *Mizuhopecten yessoensis* (*Patinopecten yessoensis*): Scallop shell matrix protein MSP-1 (C); Scallop shell matrix protein SP-S (Sarashina and Endo, 1998, 2001; Hasegawa and Uchiyama, 2005) (D); Nacrein-like protein P2 (E); Nacrein-like protein P1 (Norizuki and Samata, 2008) (F).

Figure 4. A: Decrease in THAA concentration with increasing bleaching time for modern *Pecten* (bulk powders). B: Effect of bleaching on Asx THAA D/L values in modern *Pecten* shells. Error bars (uncertainty contained within symbols) represent one standard deviation around the mean, calculated on three laboratory replicates for the same time point.

Figure 5. A: Limit of detection (LOD) compared to the concentrations of amino acids in the water used to heat (140°C for 24 hours) bleached and unbleached *Pecten*. Error bars represent one standard deviation around the mean, calculated on three laboratory replicates for the same time point. B, C, D, E: THAA D/L of Asx (B), Glx (C), Ala (D), Val (E) detected in bleached and unbleached modern *Pecten* heated at 140°C for 1 and 2 hours.

Figure 6: THAA D/L (A) and %FAA (B) values measured on bleached *Pecten* powders heated at 140°C. [Ser] values fall below LOD after 2 hours heating and therefore %FAA Ser were not calculated, although all time points are shown in Figure 6A. Error bars represent one standard deviation around the mean, calculated on three laboratory replicates for the same time point.

Figure 7: Patterns of diagenesis at high and low temperatures in bleached modern and fossil *Pecten* powders. THAA vs FAA D/L plots for Asx (A), Glx (C), Ala (E) and Val (G); THAA D/L vs %FAA plots for Asx (B), Glx (D), Ala (F) and Val (H). Val values for the 80°C experimental samples were not determined as the concentrations of FAA D-Val were too low to be detected.

Figure 8: THAA D/Ls for Asx (A), Val (B), Glx (C) and Ala (E) and THAA [Ser]/[Ala] values (D, note that y-axis values are plotted in reverse order) for fossil bleached *Pecten* shells from geological deposits of known age (U-Th dates, Table 1), Gulf of Corinth, Greece. Vertical error bars represent the expected coefficient of variation (CV) for each amino acid, calculated on the basis of the securely-dated MIS 5e shells. Horizontal error bars represent the U-Th numerical age error (Table 1). Test shell Gr9 (dark grey squares) is plotted in the mid-point of MIS 5, with horizontal error bars spanning the whole of MIS 5.